

# Measurement of Glutathione S-Transferase P1-1 in Plasma

## *Pitfalls and Significance of Screening and Follow-Up of Patients with Gastrointestinal Carcinoma*

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Presented in part at the Meeting of the Netherlands Society of Gastroenterology, Veldhoven, The Netherlands, October 3-4, 1996; published in abstract form in Mulder TPJ, Peters WHM, Wobbes TH, Witteman BJM, Jensen JBMJ. Significance of plasma glutathione S-transferase levels for screening and follow-up of patients with gastrointestinal cancer. [abstract]. *J Gastroenterol Hepatol* 1996;8:A45.

Supported by a grant from the Dutch Cancer Society (NUKC 92-33).

The authors thank all patients and blood donors participating in this study and gratefully acknowledge Dr. H. Olthuis and his staff of the Red Cross Blood Bank, Nijmegen, for collecting blood samples from the donors.

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Received November 25, 1996; revision received April 14, 1997; accepted May 9, 1997.

**BACKGROUND.** Gastrointestinal tumors often contain high amounts of the detoxification enzyme glutathione S-transferase P1-1 (GSTP1-1). Elevated levels of GSTP1-1 were found in serum and plasma from most patients with gastrointestinal tumors. The authors evaluated the role of GSTP1-1 as a plasma tumor marker in patients with gastrointestinal tumors.

**METHODS.** A sensitive and specific sandwich enzyme-linked immunosorbent assay for quantification of GSTP1-1 in human plasma was developed.

**RESULTS.** GSTP1-1 levels in serum samples from 10 healthy controls were significantly ( $P < 0.0001$ ) higher than in corresponding ethylenediaminetetraacetic acid (EDTA) plasma and varied with the type of blood collection tube used. Refrigeration or delayed centrifugation of blood collected in plain EDTA tubes resulted in spuriously high plasma GSTP1-1 levels. Therefore, all plasma samples were collected in silicone-coated EDTA tubes. The distribution of plasma GSTP1-1 levels in 230 blood donors was nearly normalized by logarithmic transformation and an upper normal reference level of 21.8  $\mu\text{g/L}$  was calculated. Males had significantly higher ( $P < 0.0001$ ) plasma GSTP1-1 levels than females and a significant increase ( $P < 0.004$ ) in plasma GSTP1-1 with age was noted. In only 20 of 55 patients (36%) with gastrointestinal tumors was the plasma GSTP1-1 level above the upper normal reference limit. No significant decrease in plasma GSTP1-1 was noted in matched pairs of plasma samples collected from 17 patients before and at least 2 weeks after resection of the tumor.

**CONCLUSIONS.** The GSTP1-1 level in serum and plasma depends on the materials and methods used to collect the samples. Only 36% of the patients with gastrointestinal tumors had elevated plasma GSTP1-1 levels that did not decrease after resection of the tumor. These findings argue against the use of GSTP1-1 as a serum or plasma marker for gastrointestinal tumors. *Cancer* 1997;80:873-80.

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**KEYWORDS:** glutathione S-transferase P1-1, tumor marker, enzyme-linked immunosorbent assay, gastrointestinal carcinoma.

**G**lutathione S-transferases (GST) (Enzyme Commission of the International Union of Biochemistry 2.5.1.18) are enzymes that catalyze the addition of glutathione to electrophilic centers of a wide variety of compounds. This reaction is the first step in the formation of mercapturic acids, a pathway mostly resulting in the elimination of potentially toxic compounds. GSTs also serve as transport proteins for a broad range of lipophilic compounds, such as bilirubin, bile acids, steroid hormones, and various xenobiotics.<sup>1,2</sup> The family of enzymes is divided into four classes: alpha,

mu, pi, and theta based on structural, physicochemical, enzymatic, and immunologic properties.<sup>1-4</sup>

Malignancies from the colon, stomach, urinary bladder, uterine cervix, and lung often contain increased amounts of GSTP1-1 when compared with the adjacent normal tissue.<sup>5</sup> In 1989 three studies were published, all indicating that a considerable number of patients with gastrointestinal malignancies had elevated serum GSTP1-1 levels.<sup>6-8</sup> High serum GSTP1-1 levels often normalized within 10-30 days after surgical removal of the malignancy.<sup>5,7</sup>

Because blood cells and platelets contain large amounts of GSTP1-1, the sampling procedures for serum or plasma are exceptionally critical. Howie et al.<sup>9</sup> described that platelet activation resulted in falsely elevated plasma GSTP1-1 levels. In a study of lung-carcinoma patients Howie et al.<sup>10</sup> therefore used blood collection tubes (Trombotect tubes; Abbott Laboratories, North Chicago, IL) containing inhibitors of platelet activation (0.6 ml 2.6% ethylenediaminetetraacetic acid [EDTA], 0.025% 2-chloroadenosine, and 7.0% procaine HCL), but their results were very similar to those obtained in serum from patients with gastrointestinal malignancies.<sup>6-8</sup> Hao et al.<sup>11</sup> sampled blood in both standard EDTA as well as in Trombotect tubes and, surprisingly, reported much lower plasma GSTP1-1 levels in the standard EDTA tubes.

In the current study the authors describe the development of a sandwich type enzyme-linked immunoadsorbent assay (ELISA) for human GSTP1-1 based on a monoclonal-catching and a polyclonal-detecting antibody. The assay was used to measure plasma and serum GSTP1-1 levels in blood samples collected under different conditions and in a variety of blood collection tubes.

## **MATERIALS AND METHODS**

Chemicals were of analytical grade and were obtained from Sigma (St. Louis, MO), unless otherwise stated.

### **Purification of GSTP1-1 and Preparation of the Antibodies**

GSTP1-1 was purified from human placenta.<sup>12</sup> Methods for production of the anti-GSTP1-1 monoclonal antibody (immunoglobulin [Ig]G2b antibodies with a  $\kappa$  light chain) were described previously.<sup>13,14</sup> Rabbits were immunized by intracutaneous injection of 25 mg GSTP1-1 in complete Freund's adjuvant and boosted twice by subcutaneous injection of 25  $\mu$ g GSTP1-1 in incomplete Freund's adjuvant. IgG was purified using Sepharose CL4B-protein A columns (Pharmacia, Uppsala, Sweden).

### **ELISA Procedure**

Assays were performed on polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands). All incubations were performed at room temperature in 100  $\mu$ L/well, unless otherwise stated. In between incubations, plates were washed 5 times with >200  $\mu$ L/well phosphate-buffered saline (PBS) supplemented with 0.05% (volume/volume [v/v]) Tween 20 detergent (PBS-T). Plates were coated overnight at 4 °C with 10  $\mu$ g/mL purified anti-GSTP1-1 monoclonal antibody in PBS and blocked with 200  $\mu$ L/well PBS-T supplemented with 1% (weight/volume [w/v]) bovine serum albumin (PBS-T-BSA) during 1 hour. Standard (0.4-100  $\mu$ g/L GSTP1-1) diluted in PBS-T supplemented with 10 mmol/L EDTA and 10% (v/v) heat-treated normal human plasma (PBS-T-EDTA-NHP) and plasma samples diluted with an equal volume of PBS-T-EDTA-NHP then were added to the wells. Plates were incubated overnight, washed, incubated with rabbit anti-GSTP1-1 antiserum diluted 1/1000 in PBS-T supplemented with 10% (v/v) heated normal human plasma for 3 hours, washed, and subsequently incubated for 2 hours with peroxidase-labeled swine antirabbit (Dakopatts, Glostrup, Denmark) diluted 1/2000 in PBS-T-BSA. After a final wash, plates were incubated with 3.75  $\mu$ mol/L o-phenylenediamine, 1 mmol/L H<sub>2</sub>O<sub>2</sub> in 24 mmol/L sodium citrate, 51 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, pH 5.0 for 15 minutes. The reaction was stopped by adding 100  $\mu$ L 2 mol/L H<sub>2</sub>SO<sub>4</sub> and absorbance was read at 492 nanometers (nm) with a background subtraction at 620 nm. All standards and samples were measured in duplicate. A four-parameter weighted logistic regression model was used to calculate standard curves and unknowns.

### **Test Samples**

Within and between-assay coefficient of variation were calculated from 5 measurements of 10 plasma samples containing between 4.0-66.5  $\mu$ g/L GSTP1-1. Human donor plasma (endogenous GSTP1-1 concentration of 24  $\mu$ g/L) was spiked with 0, 10, and 20  $\mu$ g/L GSTP1-1 and served as control samples in every assay.

Analytic recoveries were determined in serum and in EDTA, heparin, and citrate plasma samples from 10 healthy controls by spiking 1.6, 3.2, 6.3, 12.5, 25.0, and 50.0  $\mu$ g/L GSTP1-1. Plasma samples from patients with inflammatory bowel disease (n = 6) or colorectal tumors (n = 6), healthy controls (n = 6), and cytosolic fractions from normal colon mucosa (n = 5) and colorectal adenocarcinomas (n = 5), were used to evaluate whether serial dilutions of samples were parallel to the standard curve. GSTP1-1 levels in cytosolic fractions from 18 matched pairs of normal colonic mucosa and colorectal adenocarcinomas were measured by ELISA

and compared with the results obtained by densitometric analyses of immunoblots stained for GSTP1-1.<sup>15</sup>

#### Patients and Controls

EDTA plasma from 230 healthy blood donors (94 males and 136 females; median age, 35 years, range, 18–69 years) visiting the Red Cross Blood Bank Nijmegen and from 107 patients with inflammatory bowel disease (41 males and 66 females; median age, 38 years, range, 18–82 years) visiting the outpatient clinic of the Department of Gastroenterology served as controls. EDTA plasma from 55 patients with gastrointestinal malignancies, (36 males and 19 females; median age, 67 years, range, 44–95 years) was collected at the Department of Surgery and the Department of Gastroenterology, St. Radboud University Hospital, Nijmegen, The Netherlands and at the Department of Gastroenterology and Hepatology, Hospital Gelderse Vallei, Beekwijk, The Netherlands. Colorectal tumors were classified according to Dukes<sup>16</sup> as modified by Astler and Coller.<sup>17</sup> Tumors from the esophagus or stomach were staged according to the TNM classification of the International Union Against Cancer.<sup>18</sup> Of the tumors from the eight patients with esophageal carcinoma two were Stage II, three were Stage III, and three were Stage IV. Two of the five patients with gastric carcinoma had a Stage III tumor and three had a Stage IV tumor. Of the 33 colonic tumors 1 was Dukes Stage A, 6 were Dukes Stage B1, 14 were Dukes Stage B2, 7 were Dukes Stage C2, and 5 were Dukes Stage D. Of the nine rectal tumors one was Dukes Stage A, three were Dukes Stage B1, three were Dukes Stage B2 and two were Dukes Stage D. From 17 patients who underwent surgery for cure, (10 with a Dukes Stage B and 4 with a Dukes Stage C colorectal tumor and 1 with a Stage II and 2 with a Stage IV esophageal tumor) postoperative plasma samples (median, 28 days after surgery; range, 15–120 days after surgery) were also available. Informed consent was obtained from all patients and blood donors.

The patients' plasma samples were collected in silicone-coated glass tubes containing K<sub>3</sub>EDTA. Immediately after collection of the sample, blood was gently mixed with the EDTA solution. Tubes were centrifuged for 10 minutes at 3000 × g at room temperature within 2 hours. The upper two-thirds of the plasma was collected and care was taken not to aspirate the platelets on top of the cell layer. Plasma samples were stored in portions at –20 °C.

#### Miscellaneous

Blood collection tubes were obtained from Becton Dickinson (Grenoble, France) and from Sherwood

Medical (Ballymoney, Northern Ireland). Table 1 provides further specifications. The enzyme immunoassay for platelet factor 4 (PF4) was obtained from Stago Diagnostica (Asnières-sur-Seine, France).

Amounts of GST protein in standard preparations were quantified by the Biorad protein assay (Biorad Laboratories, Munich, Germany) using a BSA standard line.

Human plasma was depleted of all immunoreactive GSTP1-1 by heating at 60 °C for 30 minutes.<sup>10</sup>

GST enzyme activity was assayed by the method of Habig et al.<sup>19</sup> using 1-chloro-2,4-dinitrobenzene as substrate. Specific activity of the GSTP1-1 standard preparation was 112 μmol/minute · mg protein.

To evaluate the significance of differences between two groups, the Mann-Whitney *U* test or the Wilcoxon matched-pairs signed rank test was used. Differences between more than two groups were evaluated by Kruskal-Wallis analysis.

GSTP1-1 levels are expressed as median (range), unless otherwise stated.

This study was approved by the local Medical Ethical Review Committee.

## RESULTS

### Assay Performance

Coating concentration of the monoclonal antibody and dilutions of the rabbit antihuman GSTP1-1 antiserum and the swine antirabbit peroxidase were optimized at 10 mg/L, 1/1000 and 1/2000, respectively. The detection limit of the resulting assay, corresponding to 3 standard deviations above the mean signal of 5 zero standards in duplicate, was 0.4 μg/L. GST-α and GST-μ in concentrations up to 10 mg/L displayed no detectable cross-reactivity.

Recoveries in spiked serum, citrate, heparin, or EDTA plasma were 80%, 86%, 82%, and 82%, respectively. Twofold dilution of plasma samples from healthy controls and various patient populations or twofold dilutions of cytosolic fractions from normal colonic mucosa and colonic tumors were all parallel to the standard curve.

Within- and between-assay coefficients of variation ranged from 1.3–10.2% (mean, 5.8%) and from 7.4–15.2% (mean, 10.9%), respectively. GSTP1-1 levels were also measured using both the ELISA and the semi-quantitative immunoblot assay in cytosolic fractions from 18 matched pairs of normal colonic mucosa and colorectal tumors. A good linear correlation (correlation coefficient [*r*] = 0.85) was obtained between the two assays.

### Influence of Sample Collection on GSTP1-1 Levels

GSTP1-1 levels in serum samples from ten healthy controls were higher than levels in the corresponding

TABLE 1  
Glutathione S-transferase P1-1 Levels in Plasma and Serum, Collected in Different Tubes

Control no.	GSTP1-1 $\mu\text{g/L}$							
	Plasma					Serum		
	A	B	C	D	E	F	G	H
1	5.8	4.8	4.6	2.8	4.2	20.4	28.2	17.4
2	5.8	7.0	5.4	4.2	5.0	11.0	16.8	8.6
3	5.2	4.8	5.4	5.0	4.6	13.6	29.8	14.4
4	5.6	6.0	6.4	5.4	6.2	11.6	23.8	14.2
5	5.8	5.2	5.4	5.2	5.0	17.6	21.8	15.6
6	8.2	6.0	7.2	5.6	5.6	15.4	32.4	13.6
7	10.6	7.2	6.4	6.2	8.2	17.2	32.0	18.0
8	9.2	5.0	5.2	10.0	6.4	12.0	6.8	9.4
9	16.6	10.2	9.0	11.0	13.2	43.6	28.2	17.2
10	7.4	5.4	4.6	4.4	6.6	12.2	21.2	11.2
Median	6.6 <sup>a</sup>	5.7	5.4	5.3	5.9	14.5 <sup>a</sup>	26.0 <sup>a</sup>	14.3 <sup>a</sup>
Range	5.2–16.6	4.8–10.2	4.6–9.0	2.8–11.0	4.2–13.2	11.0–43.6	6.8–32.4	8.6–18.0

GSTP1-1: glutathione S-transferase P1-1.

<sup>a</sup> $P < 0.01$  vs. Tube C.

Plasma and serum glutathione S-transferase P1-1 levels in ten healthy controls as determined by enzyme-linked immunosorbent assay after collection of blood in eight different vacuum tubes. After venipuncture tubes were filled in random order. All blood samples were stored at room temperature for 1 hour and centrifuged at  $3000 \times g$  for 10 minutes at room temperature. Plasma or serum was subsequently stored at  $-20^\circ\text{C}$  until analysis.

Tube A: Sherwood Medical Monoject (Ballymoney, Northern Ireland) uncoated glass tube,  $16 \times 100$  mm; additive: 0.10 mL 15%  $\text{K}_2\text{EDTA}$  (Order no. 1000-311743).

Tube B: Becton Dickinson Vacutainer, (Grenoble, France) uncoated glass tube  $13 \times 75$  mm; additive: 0.054 mL 15%  $\text{K}_2\text{EDTA}$  (Order no. 367654).

Tube C: Becton Dickinson Vacutainer, silicone-coated glass tube,  $16 \times 75$  mm; additive: 0.048 mL 15%  $\text{K}_2\text{EDTA}$  (Order no. 606601).

Tube D: Sherwood Medical Monoject, silicone-coated glass tube,  $12 \times 75$  mm; additive: 0.06 mL 7.5%  $\text{K}_2\text{EDTA}$  (Order no. 1000-311000).

Tube E: Becton Dickinson Vacutainer Diatube H, glass tube with unknown coating,  $12 \times 75$  mm; additives: citrate, theophylline, adenosine, and dipyrimidine (Order no. 367015).

Tube F: Sherwood Medical Monoject, silicone-coated glass tube,  $16 \times 100$  mm; additive: none (Order no. 1000-301710).

Tube G: Sherwood Medical Corvac, silicone-coated glass tube,  $16 \times 100$  mm; additives: powdered glass (cloth activator), inert plastic (energizer), and thixotropic (gel) (Order no. 8881-302015).

Tube H: Becton Dickinson Vacutainer, glass tube with unknown coating,  $13 \times 100$  mm; additives: silica (cloth activator) and polyester (gel) (Order no. 367784).

EDTA plasma samples (Table 1). Moreover, serum GSTP1-1 values varied with the blood collection tube used. GSTP1-1 concentrations measured in EDTA plasma collected in different brands of blood collection tubes were more consistent (Table 1). When blood was sampled in plain EDTA tubes (Tube A) and centrifuged at  $4^\circ\text{C}$ , high plasma GSTP1-1 levels were found ( $28 \mu\text{g/L}$ ;  $11.8$ – $55.2 \mu\text{g/L}$ ) when compared with samples centrifuged at room temperature ( $6.6 \mu\text{g/L}$ ;  $5.2$ – $16.6 \mu\text{g/L}$ ). In plasma sampled in EDTA tubes with a silicone coating (Tube C), no significant effect of refrigeration was noted ( $6.0 \mu\text{g/L}$  [ $4.4$ – $8.2 \mu\text{g/L}$ ] vs.  $5.4 \mu\text{g/L}$  [ $4.6$ – $9.0 \mu\text{g/L}$ ]), when centrifuged at  $4^\circ\text{C}$  and at room temperature, respectively. Plasma GSTP1-1 levels in blood samples collected in plain, uncoated EDTA tubes (Tube A) increased from  $6.6 \mu\text{g/L}$  ( $5.2$ – $16.6 \mu\text{g/L}$ ) to  $13.3 \mu\text{g/L}$  ( $10.8$ – $33.6 \mu\text{g/L}$ ) when tubes were centrifuged 1 and 6 hours after sampling, respectively. In silicone-coated tubes (Tube C), no significant effect of delayed centrifugation was noted ( $5.4 \mu\text{g/L}$  [ $4.6$ – $9.0 \mu\text{g/L}$ ] vs.  $5.3 \mu\text{g/L}$  [ $4.2$ – $9.0 \mu\text{g/L}$ ]) after storage for 1 and 6 hours at room temperature, respectively.

However, mild shaking of the silicone-coated EDTA tubes (Tube C) during 1 hour resulted in elevated GSTP1-1 levels ( $14.6 \mu\text{g/L}$  [ $11.8$ – $19.0 \mu\text{g/L}$ ]). GSTP1-1 values measured in plasma collected in EDTA tubes (Tubes A–D) were very similar to values obtained in samples collected in tubes containing citrate, theophylline, adenosine, and dipyrimidine (CTAD) (Tube E). However, CTAD plasma contained much lower levels of PF4 ( $30 \text{ IU/mL}$  [ $17$ – $64 \text{ IU/mL}$ ]) vs.  $284 \text{ IU/mL}$  [ $159$ – $578 \text{ IU/mL}$ ] for plasma collected in EDTA tubes (Tube C), indicating that even in silicone-coated EDTA tubes platelets had been activated.

#### GSTP1-1 in EDTA Plasma from Patients and Controls

Subsequently, all samples were collected in the same type of silicone-coated EDTA tubes (Tube C). Plasma GSTP1-1 levels in 230 healthy controls displayed a leptokurtic positively skewed distribution that could be nearly normalized by logarithmic transformation. On the logarithmic scale the mean value was  $7.8 \mu\text{g/L}$  and the reference range (mean  $\pm 2$  standard deviations) was  $2.8$ – $21.8 \mu\text{g}$  GSTP1-1 per liter of EDTA plasma.

**TABLE 2**  
Plasma Glutathione S-transferase P1-1 Concentrations in Healthy Controls Divided According to Gender and Age

Age group (yrs)	$\mu\text{g/L}$ GSTP1-1, median (range), (no. of controls)		
	Males	Females	All
20-40	8.2 (3.8-33.6) <sup>a</sup> (41)	5.5 (2.3-31.5) <sup>e</sup> (81)	6.5 (2.3-33.6) <sup>g</sup> (122)
40-60	9.1 (5.8-17.6) <sup>b</sup> (32)	6.6 (4.0-71.6) <sup>f</sup> (40)	7.8 (4.0-71.6) <sup>h</sup> (72)
>60	12.0 (7.2-25.2) <sup>c</sup> (20)	11.00 (5.1-24.2) (12)	11.8 (5.1-25.2) (32)
All ages	9.1 (3.8-33.6) <sup>d</sup> (94)	6.2 (2.2-71.6) (136)	7.2 (2.2-71.6) (230)

GSTP1-1: glutathione S-transferase P1-1.

<sup>a</sup>Not significant vs. males 40-60 years;  $P = 0.0018$  vs. males >60 years;  $P < 0.0001$  vs. females 20-40 years.

<sup>b</sup> $P = 0.00187$  vs. males >60 years;  $P = 0.0004$  vs. females >40-60 years.

<sup>c</sup>Not significant vs. females >60 years.

<sup>d</sup> $P < 0.0001$  vs. all females.

<sup>e</sup> $P = 0.0406$  vs. females 40-60 years;  $P = 0.0002$  vs. females >60 years.

<sup>f</sup> $P = 0.0320$  vs. females >60 years.

<sup>g</sup>Not significant vs. 40-60 years;  $P < 0.0001$  vs. >60 years.

<sup>h</sup> $P < 0.0001$  vs. >60 years.

Ages of three females and one male were not recorded and therefore the numbers do not add up.

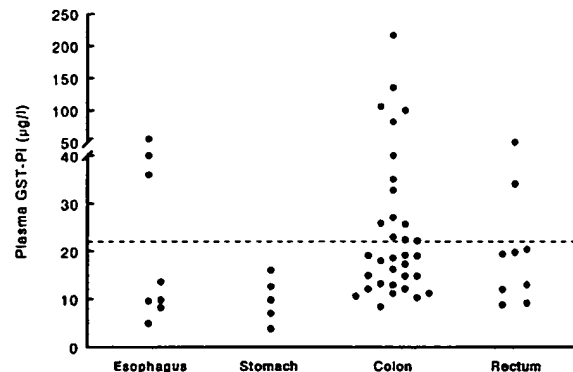
Males had significantly ( $P < 0.0001$ ) higher plasma GSTP1-1 levels than females and a significant ( $P < 0.004$ ) increase with age was noted both in males and females (Table 2). Patients with inflammatory bowel disease had slightly, but significantly ( $P < 0.02$ ), lower plasma GSTP1-1 values ( $6.8 \mu\text{g/L}$ ;  $1.6-23.0 \mu\text{g/L}$ ;  $n = 107$ ) than healthy controls ( $7.2 \mu\text{g/L}$ ;  $2.2-71.6 \mu\text{g/L}$ ;  $n = 230$ ).

When compared with the healthy controls, patients with gastrointestinal tumors had significantly ( $P < 0.0001$ ) elevated plasma GSTP1-1 levels ( $20.4 \mu\text{g/L}$ ;  $8.2-216.8 \mu\text{g/L}$ ) and in 20 of the 55 patients (36%) plasma GSTP1-1 levels were above the upper normal reference limit (Fig. 1). However, plasma GSTP1-1 levels were not higher in patients with more advanced stages of colorectal carcinoma (Table 3) and no significant effect of surgery was noted in 17 patients with a gastrointestinal tumor for whom plasma samples before and after resection of the tumor were available (Fig. 2).

## DISCUSSION

### Assay

The sandwich ELISA used in the current study had a detection limit and an intra- and interassay coefficient of variation comparable to previously published immunoassays for GSTP1-1.<sup>6-9</sup> Recoveries of spiked GSTP1-1 in several types of plasma and in serum were



**FIGURE 1.** Plasma glutathione S-transferase P1-1 (GSTP1-1) levels in patients with various gastrointestinal malignancies. The dashed line denotes the upper normal reference limit.

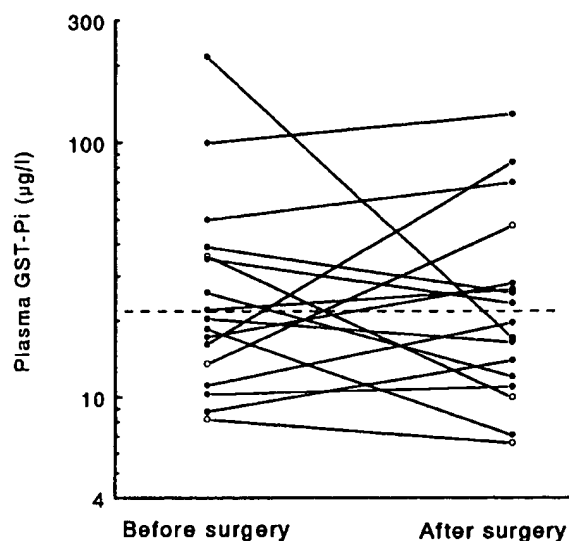
>80% and serial dilutions of plasma and serum samples and dilutions of tissue homogenates were parallel to the standard curve. No significant cross-reactivity with GST-alpha or GST-mu (up to 10 mg/L) was observed. In addition, results obtained in tissue homogenates were comparable to levels obtained using the authors' semiquantitative immunoblot assay. All these findings indicate that the measurements of GSTP1-1 in plasma, serum, and tissue homogenates obtained by the authors were accurate.

**TABLE 3**  
Plasma Glutathione S-Transferase P1-1 Concentrations in Patients with Colorectal Carcinoma According to Stage of the Tumor

Tumor stage	$\mu\text{g/L}$ GSTP1-1, median (range), (no. of patients)
Dukes A	21.1 (19.8–22.4) (2)
Dukes B	21.3 (8.4–216.8) (26)
Dukes C	18.6 (10.3–105.8) (9)
Dukes D	14.8 (12.1–32.8) (5)

GSTP1-1: glutathione S-transferase P1-1.

Differences between groups were not significant.



**FIGURE 2.** Plasma glutathione S-transferase P1-1 (GSTP1-1) levels in patients with carcinoma of the esophagus (open circles) or colorectum (closed circles). Samples were taken a few days before and >14 days after surgery. The dashed line denotes the upper normal reference limit.

#### Collection of Samples

Hida et al.<sup>20</sup> compared GSTP1-1 levels in serum with those obtained in EDTA plasma from 50 patients. They reported a significant linear correlation ( $r = 0.81$ ) between serum and plasma GSTP1-1 levels, but no further information was provided. Platelets contain major amounts of GSTP1-1 and the release of GSTP1-1 by activated platelets may lead to spuriously high GSTP1-1 levels in serum and plasma.<sup>9</sup> In a subsequent study Howie et al.<sup>10</sup> analyzed plasma and serum samples from eight controls and reported that serum contained

GSTP1-1 levels of up to three times the upper normal reference limit for plasma. In the current study a similar difference between serum and plasma GSTP1-1 levels was detected. Moreover, the authors also detected major differences in serum GSTP1-1 levels between serum samples collected in various types of tubes.

Howie et al.<sup>10</sup> claimed that Trombotect tubes obviate spuriously high results of GSTP1-1 in plasma by inhibiting platelet activation. To quantify platelet activation, they measured PF4, a protein secreted from the  $\alpha$  granules of stimulated blood platelets. Hao et al.<sup>11</sup> also used Trombotect tubes but in their hands plasma collected in these tubes contained approximately fourfold to tenfold more GSTP1-1 than plasma collected in standard EDTA tubes, whereas similar levels of PF4 were measured in both sets of samples. Trombotect tubes were not for sale in The Netherlands and the authors used CTAD tubes, which were also developed to prevent platelet activation. In the authors' hands plasma GSTP1-1 levels in these tubes were similar as in EDTA tubes, although PF4 levels were almost tenfold higher in EDTA plasma. These data, together with the results from Hao et al.,<sup>11</sup> suggest that PF4 is an inadequate marker for GSTP1-1 release from blood platelets.

Because EDTA tubes provided the lowest plasma GSTP1-1 levels in healthy control subjects, the authors initially collected plasma samples in standard (uncoated glass) EDTA tubes. Although most samples contained normal levels of GSTP1-1, occasional high levels were found, even in healthy controls. Subsequent experiments indicated that cooling the tubes to 4 °C before or during centrifugation increased GSTP1-1 levels in the resulting plasma almost threefold. A significant rise in plasma GSTP1-1 levels was also noted when centrifugation was delayed for several hours. Howie et al.<sup>10</sup> previously reported that GSTP1-1 levels in plasma samples collected in Trombotect tubes increased within 2–3 hours after sampling. The authors of the current study concluded that plasma sampled in uncoated EDTA tubes was not suitable for plasma GSTP1-1 measurements. In silicone-coated EDTA tubes no increase in plasma GSTP1-1 levels due to cooling or delayed centrifugation of the tubes was noted. Fan et al.<sup>21</sup> also used silicone-coated EDTA tubes, but they also added procaine HCL to inhibit platelet activation.

The results of the current study indicate that even minor variations in the type of blood collection tubes or in the procedures used to handle and centrifuge the blood and the subsequent aspiration of plasma samples may substantially affect the resulting GSTP1-1 levels. Therefore, only GSTP1-1 levels measured in samples collected in the same type of blood collection

tubes and processed under identical conditions can be compared.

#### Reference Levels

Upper normal reference limits of 3.7  $\mu\text{g/L}$ ,<sup>8</sup> 15.4  $\mu\text{g/L}$ ,<sup>7</sup> 34.0  $\mu\text{g/L}$ ,<sup>6</sup> and 34.8  $\mu\text{g/L}$ <sup>20</sup> GSTP1-1 in serum, 30.4  $\mu\text{g/L}$  GSTP1-1 in heparin plasma,<sup>22</sup> and 5.4  $\mu\text{g/L}$ <sup>11</sup> and 13.99  $\mu\text{g/L}$ <sup>21</sup> in EDTA plasma were reported in the literature, based on samples from 30–117 healthy controls. In none of these studies were differences between males and females or different age groups noted, and in all except one of the studies a normal distribution was assumed. Only Takahashi et al.<sup>6</sup> reported that their normal reference values, based on 114 healthy controls, had to be log-transformed to obtain a nearly normal distribution. These authors also demonstrated a slight increase in the serum GSTP1-1 levels after age 70 years. The results of the current study in 230 healthy controls indicated that males had significantly higher GSTP1-1 levels in EDTA plasma than females. In both males and females a significant increase was noted with increasing age. In agreement with the results reported by Takahashi et al.<sup>6</sup> the current data had to be log-transformed to obtain a nearly normal distribution.

The authors also studied the plasma GSTP1-1 values in a large group of patients with inflammatory bowel disease, a disorder characterized by chronic, recurrent inflammation of the intestinal mucosa. GSTP1-1 levels in intestinal mucosa are relatively high and due to the process of inflammation some leakage of GSTP1-1 from the mucosa to the blood may occur, leading to increased plasma GSTP1-1 levels in these patients. However, slightly lower plasma levels were detected in patients with inflammatory bowel disease.

#### Patients with Gastrointestinal Tumors

In the current study, 36% (20 of 55) of the patients with esophageal, gastric, or colorectal carcinoma had elevated plasma GSTP1-1 values. In the study by Fan et al.<sup>21</sup> 73% (70 of 96) of the patients with gastrointestinal tumors had elevated plasma GSTP1-1 levels. Corresponding figures obtained in serum samples were 48% (40 of 83), 52% (39 of 75), and 64% (66 of 103) in the studies by Takahashi et al.,<sup>6</sup> Tsuchida et al.,<sup>8</sup> and Niitsu et al.,<sup>7</sup> respectively.

The relatively low percentage of patients with elevated GSTP1-1 levels in the current study may be related to the large percentage of patients with colorectal tumors (42 of 55; 76%). In the studies by Takahashi et al.,<sup>6</sup> Tsuchida et al.,<sup>8</sup> Niitsu et al.,<sup>7</sup> and Fan et al.<sup>21</sup> the majority of patients studied had gastric carcinoma. The lowest percentages of tumor patients with elevated plasma GSTP1-1 levels were found by Takahashi

et al.<sup>6</sup> and in the current study. This may be related to the logarithmic transformation of the normal reference values that was performed to calculate the upper normal reference limit. This transformation leads to a higher upper normal reference limit and consequently to a lower percentage of patients with elevated serum or plasma GSTP1-1 levels. Preliminary results in patients with oral carcinoma (8 of 56 patients; 14%; Oude Ophuis et al., unpublished data) or bladder carcinoma (10 of 40 patients; 25%; Berendsen et al., unpublished data) also indicated that only a small percentage had elevated plasma GSTP1-1 levels. Overall positivity of plasma/serum GSTP1-1 is comparable to other serum/plasma markers for gastrointestinal malignancies such as carcinoembryonic antigen, CA 50, CA 19-9, CA 195, or others,<sup>23,24</sup> which were also elevated in 30–60% of the patients.

Niitsu et al.<sup>7</sup> and Fan et al.<sup>21</sup> both reported that plasma or serum GSTP1-1 levels in patients with gastric carcinoma were higher in patients with more advanced tumor stages. Similar results were reported for patients with nonsmall cell lung carcinoma and patients with oral carcinoma.<sup>22</sup> In the group of patients with colorectal carcinoma studied by the authors, no significant relation between tumor stages and plasma GSTP1-1 levels was noticed.

Hirata et al.<sup>22</sup> studied patients with oral carcinoma and reported an impressive decline in plasma GSTP1-1 levels after surgical resection of the tumor; 16 of 25 patients with a good prognosis had elevated GSTP1-1 levels before surgery and all values had normalized 8 weeks postoperatively. Similar investigations in patients with gastrointestinal tumors did not provide such striking results. Niitsu et al.<sup>7</sup> reported a gradual decrease of serum GSTP1-1 levels in two patients with gastric carcinoma after resection. After 10 days serum GSTP1-1 levels had returned to values within the normal reference range. Fan et al.<sup>21</sup> studied plasma GSTP1-1 levels in 18 patients with colorectal carcinoma. In 8 of the 14 patients with an elevated preoperative value (57%) they detected normal plasma GSTP1-1 levels within 14 days after surgery. Nineteen of the 29 patients with esophageal carcinoma studied by Tsuchida et al.<sup>8</sup> had an elevated preoperative serum GSTP1-1 level. In the serum of 9 of these 19 patients (47%) normal GSTP1-1 levels were measured after resection of the tumor. The authors studied 17 patients with gastrointestinal carcinomas before and after surgical resection of the tumor. Eight patients had elevated plasma GSTP1-1 levels before surgery and in 3 of these patients (38%) these levels were within the normal range after removal of the tumor. However, of the 9 patients with normal preoperative plasma GSTP1-1 levels, 3 (33%) demonstrated elevated post-

operative levels. Thus, the number of patients with elevated plasma GSTP1-1 remained unchanged after resection of the tumor, indicating that high plasma GSTP1-1 levels were not caused by leakage from the primary tumor.

The concentration of GSTP1-1 in serum and plasma samples depends mainly on the materials and methods used to collect the samples. In the current study siliconized EDTA blood collection tubes gave the lowest GSTP1-1 levels in healthy controls. Although > 33% of the patients with gastrointestinal tumors had elevated plasma GSTP1-1 levels, follow-up experiments after resection of the tumor indicated that the increased GSTP1-1 levels in plasma may not be derived from the tumor. Combined with the high probability of generating false-positive results due to inappropriate sampling, the findings of the current study argue against the use of GSTP1-1 as a routine plasma or serum tumor marker.

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